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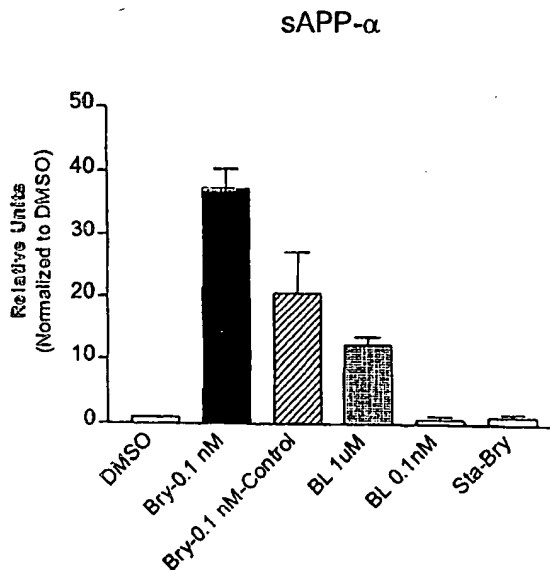
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(54) Title: PKC ACTIVATION AS A MEANS FOR ENHANCING sAPP α SECRETION AND IMPROVING COGNITION USING BRYOSTATIN TYPE COMPOUNDS



(57) Abstract: The present invention relates to compositions and methods to modulate α -secretase and/or to improve cognitive ability. The invention further relates the improved/enhanced cognitive ability in diseased individuals, particularly Alzheimer's Disease patients, and treatment thereof through increased sAPP production. Macrocyclic lactones (i.e. bryostatin class and neristatin class) are compounds preferred for use with the present composition. The present invention also provides methods for increasing the generation of non-amyloidogenic soluble APP comprising the activation of protein kinase C (PKC) by administering an effective amount of PKC activator(s).

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

PKC Activation as a Means for Enhancing sAPP α Secretion and Improving Cognition Using Bryostatin Type Compounds

BACKGROUND OF THE INVENTION

(i) Field of the Invention

5 The present invention relates to the modulation of α -secretase and cognitive enhancement. The invention further relates to compounds for treatment of conditions associated with amyloid processing such as Alzheimer's Disease and compositions for the treatment of such conditions.

(ii) Background of the Invention

10 Various disorders and diseases exist which affect cognition. Cognition can be generally described as including at least three different components: attention, learning, and memory. Each of these components and their respective levels affect the overall level of a subject's cognitive ability. For instance, while Alzheimer's Disease patients suffer from a loss of overall cognition and thus deterioration of each
15 of these characteristics, it is the loss of memory that is most often associated with the disease. In other diseases patients suffer from cognitive impairment that is more predominately associated with different characteristics of cognition. For instance Attention Deficit Hyperactivity Disorder (ADHD), focuses on the individual's ability to maintain an attentive state. Other conditions include general dementias
20 associated with other neurological diseases, aging, and treatment of conditions that can cause deleterious effects on mental capacity, such as cancer treatments, stroke/ischemia, and mental retardation.

Cognition disorders create a variety of problems for today's society. Therefore, scientists have made efforts to develop cognitive enhancers or cognition
25 activators. The cognition enhancers or activators that have been developed are generally classified to include nootropics, vasodilators, metabolic enhancers, psychostimulants, cholinergic agents, biogenic amine drugs, and neuropeptides. Vasodilators and metabolic enhancers (e.g. dihydroergotoxine) are mainly effective in the cognition disorders induced by cerebral vessel ligation-ischemia; however,
30 they are ineffective in clinical use and with other types of cognition disorders. Of

the developed cognition enhancers, typically only metabolic drugs are employed for clinical use, as others are still in the investigation stage. Of the nootropics for instance, piracetam activates the peripheral endocrine system, which is not appropriate for Alzheimer's disease due to the high concentration of steroids
5 produced in patients while tacrine, a cholinergic agent, has a variety of side effects including vomiting, diarrhea, and hepatotoxicity.

Ways to improve the cognitive abilities of diseased individuals have been the subject of various studies. Recently the cognitive state related to Alzheimer's Disease and different ways to improve patient's memory have been the subject of
10 various approaches and strategies. Unfortunately, these approaches and strategies only improve symptomatic and transient cognition in diseased individuals but have not addressed the progression of the disease. In the case of Alzheimer's Disease, efforts to improve cognition, typically through the cholinergic pathways or through other brain transmitter pathways, have been investigated. The primary approach
15 relies on the inhibition of acetyl cholinesterase enzymes through drug therapy. Acetyl cholinesterase is a major brain enzyme and manipulating its levels can result in various changes to other neurological functions and cause side effects.

While these and other methods may improve cognition, at least transiently, they do not modify the disease progression, or address the cause of the disease. For
20 instance, Alzheimer's Disease is typically associated with the formation of plaques through the accumulation of amyloid precursor protein. Attempts to illicit an immunological response through treatment against amyloid and plaque formation have been done in animal models, but have not been successfully extended to humans.

25 Furthermore, cholinesterase inhibitors only produce some symptomatic improvement for a short time and in only a fraction of the Alzheimer's Disease patients with mid to moderate symptoms and are thus only a useful treatment for a small portion of the overall patient population. Even more critical is that present efforts at improving cognition do not result in treatment of the disease condition, but
30 are merely ameliorative of the symptoms. Current treatments do not modify the disease progression. These treatments have also included the use of a "vaccine" to

treat the symptoms of Alzheimer's Disease patients which, while theoretically plausible and effective in mice tests, have been shown to cause severe adverse reactions in humans.

As a result, use of the cholinergic pathway for the treatment of cognitive impairment, particularly in Alzheimer's Disease, has proven to be inadequate. Additionally, the current treatments for cognitive improvement are limited to specific neurodegenerative diseases and have not proven effective in the treatment of other cognitive conditions.

Alzheimer's disease is associated with extensive loss of specific neuronal subpopulations in the brain with memory loss being the most universal symptom. (Katzman, R. (1986) New England Journal of Medicine 314:964). Alzheimer's disease is well characterized with regard to neuropathological changes. However, abnormalities have been reported in peripheral tissue supporting the possibility that Alzheimer's disease is a systemic disorder with pathology of the central nervous system being the most prominent. (Connolly, G., Fibroblast models of neurological disorders: fluorescence measurement studies, Review, TiPS Vol. 19, 171-77 (1998)). For a discussion of Alzheimer's disease links to a genetic origin and chromosomes 1, 14, and 21 see St. George-Hyslop, P. H., et al., Science 235:885 (1987); Tanzi, Rudolph et al., The Gene Defects Responsible for Familial Alzheimer's Disease, Review, Neurobiology of Disease 3, 159-168 (1996); Hardy, J., Molecular genetics of Alzheimer's disease, Acta Neurol Scand: Supplement 165: 13-17 (1996).

While cellular changes leading to neuronal loss and the underlying etiology of the disease remain under investigation the importance of APP metabolism is well established. The two proteins most consistently identified in the brains of patients with Alzheimer's disease to play a role in the physiology or pathophysiology of brain are β -amyloid and tau. (See Selkoe, D., Alzheimer's Disease: Genes, Proteins, and Therapy, Physiological Reviews, Vol. 81, No. 2, 2001). A discussion of the defects in β -amyloid protein metabolism and abnormal calcium homeostasis and/or calcium activated kinases. (Etcheberrigaray et al., Calcium responses are altered in fibroblasts from Alzheimer's patients and pre-symptomatic PS1 carriers: a potential tool for early diagnosis, Alzheimer's Reports, Vol. 3, Nos. 5 & 6, pp. 305-312

(2000); Webb et al., Protein kinase C isozymes: a review of their structure, regulation and role in regulating airways smooth muscle tone and mitogenesis, British Journal of Pharmacology, 130, pp 1433-52 (2000)).

Further with regard to normal and abnormal memory both K^+ and Ca^{2+} channels have been demonstrated to play key roles in memory storage and recall. For instance, potassium channels have been found to change during memory storage. (Etcheberrigaray, R., et al. (1992) Proceeding of the National Academy of Science 89:7184; Sanchez-Andres, J. V. and Alkon, D. L. (1991) Journal of Neurobiology 65:796; Collin, C., et al. (1988) Biophysics Journal 55:955; Alkon, D. L., et al. (1985) Behavioral and Neural Biology 44:278; Alkon, D. L. (1984) Science 226:1037). This observation, coupled with the almost universal symptom of memory loss in Alzheimer's patients, led to the investigation of potassium channel function as a possible site of Alzheimer's disease pathology and the effect of PKC modulation on cognition.

PKC was identified as one of the largest gene families of non-receptor serine-threonine protein kinases. Since the discovery of PKC in the early eighties by Nishizuka and coworkers (Kikkawa et al., *J. Biol. Chem.*, 257, 13341 (1982), and its identification as a major receptor for phorbol esters (Ashendel et al., *Cancer Res.*, 43, 4333 (1983)), a multitude of physiological signaling mechanisms have been ascribed to this enzyme. The intense interest in PKC stems from its unique ability to be activated *in vitro* by calcium and diacylglycerol (and its phorbol ester mimetics), an effector whose formation is coupled to phospholipid turnover by the action of growth and differentiation factors.

The PKC gene family consists presently of 11 genes which are divided into four subgroups: 1) classical PKC α , β_1 , β_2 (β_1 and β_2 are alternatively spliced forms of the same gene) and γ , 2) novel PKC δ , ϵ , η and θ , 3) atypical PKC ζ , λ , ι and 4) PKC μ . PKC μ resembles the novel PKC isoforms but differs by having a putative transmembrane domain (reviewed by Blohe et al., *Cancer Metast. Rev.*, 13, 411 (1994); Ilug et al., *Biochem j.*, 291, 329 (1993); Kikkawa et al., *Ann. Rev. Biochem.* 58, 31 (1989)). The α , β_1 , β_2 , and γ isoforms are Ca^{2+} , phospholipid and diacylglycerol-dependent and represent the classical isoforms of PKC, whereas the

other isoforms are activated by phospholipid and diacylglycerol but are not dependent on Ca^{2+} . All isoforms encompass 5 variable (V1-V5) regions, and the α , β , γ isoforms contain four (C1-C4) structural domains which are highly conserved. All isoforms except PKC α , β and γ lack the C2 domain, and the λ , η and isoforms also lack nine of two cysteine-rich zinc finger domains in C1 to which diacylglycerol binds. The C1 domain also contains the pseudosubstrate sequence which is highly conserved among all isoforms, and which serves an autoregulatory function by blocking the substrate-binding site to produce an inactive conformation of the enzyme (House et al., *Science*, 238, 1726 (1987)).

Because of these structural features, diverse PKC isoforms are thought to have highly specialized roles in signal transduction in response to physiological stimuli (Nishizuka, *Cancer*, 10, 1892 (1989)), as well as in neoplastic transformation and differentiation (Glazer, *Protein Kinase C*, J. F. Kuo, ed., Oxford U. Press (1994) at pages 171-198). For a discussion of known PKC modulators see PCT/US97/08141, U.S. Patent Nos. 5,652,232; 6,043,270; 6,080,784; 5,891,906; 5,962,498; 5,955,501; 5,891,870 and 5,962,504.

In view of the central role that PKC plays in signal transduction, PKC has proven to be an exciting target for the modulation of APP processing. It is well established that PKC plays a role in APP processing. Phorbol esters for instance have been shown to significantly increase the relative amount of non-amyloidogenic soluble APP (sAPP) secreted through PKC activation. Activation of PKC by phorbol ester does not appear to result in a direct phosphorylation of the APP molecule, however. Irrespective of the precise site of action, phorbol-induced PKC activation results in an enhanced or favored α -secretase, non-amyloidogenic pathway. Therefore PKC activation is an attractive approach for influencing the production of non-deleterious sAPP and even producing beneficial sAPP and at the same time reduce the relative amount of $A\beta$ peptides. Phorbol esters, however, are not suitable compounds for eventual drug development because of their tumor promotion activity. (Ibarreta, et al., Benzolactam (BL) enhances sAPP secretion in fibroblasts and in PC12 cells, *NeuroReport*, Vol. 10, No. 5&6, pp 1035-40 (1999)).

There is increasing evidence that the individual PKC isozymes play different,

sometimes opposing, roles in biological processes, providing two directions for pharmacological exploitation. One is the design of specific (preferably, isozyme specific) inhibitors of PKC. This approach is complicated by the fact that the catalytic domain is not the domain primarily responsible for the isotype specificity of PKC. The other approach is to develop isozyme-selective, regulatory site-directed PKC activators. These may provide a way to override the effect of other signal transduction pathways with opposite biological effects. Alternatively, by inducing down-regulation of PKC after acute activation, PKC activators may cause long term antagonism. Bryostatin is currently in clinical trials as an anti-cancer agent. The bryostatins are known to bind to the regulatory domain of PKC and to activate the enzyme. Bryostatin is an example of isozyme-selective activators of PKC. Compounds in addition to bryostatins have been found to modulate PKC. (see for example WO 97/43268)

There still exists a need for the development of methods for the treatment for improved overall cognition, either through a specific characteristic of cognitive ability or general cognition. There also still exists a need for the development of methods for the improvement of cognitive enhancement whether or not it is related to specific disease state or cognitive disorder. The methods and compositions of the present invention fulfill these needs and will greatly improve the clinical treatment for Alzheimer's disease and other neurodegenerative diseases, as well as, provide for improved cognitive enhancement. The methods and compositions also provide treatment and/or enhancement of the cognitive state through the modulation of α -secretase.

SUMMARY OF THE INVENTION

The invention relates to compounds, compositions, and methods for the treatment of conditions associated with enhancement/improvement of cognitive ability. In a preferred embodiment, the present invention further relates to compounds, compositions and methods for the treatment of conditions associated with amyloid processing, such as Alzheimer's Disease, which provides for improved/enhanced cognitive ability in the subject treated. In particular the

compounds and compositions of the present invention are selected from macrocyclic lactones of the bryostatin and neristatin class.

In another aspect the invention relates to macrocyclic lactone compounds, compositions and methods that modulate α -secretase activity. Of particular interest
5 are the bryostatin and neristatin class compounds, and of further interest is bryostatin-1.

Another aspect of the invention relates to the bryostatin and neristatin class compounds, as a PKC activator, to alter conditions associated with amyloid processing in order to enhance the α -secretase pathway to generate soluble α -
10 amyloid precursor protein (α APP) so as to prevent β -amyloid aggregation and improve/enhance cognitive ability. Such activation, for example, can be employed in the treatment of Alzheimer's Disease, particularly, bryostatin-1.

In another aspect, the invention relates to a method for treating plaque formation, such as that associated with Alzheimer's Disease, and
15 improving/enhancing the cognitive state of the subject comprising administering to the subject an effective amount of a bryostatin or neristatin class compound. In a more preferred embodiment the compound is bryostatin-1.

Another aspect of the invention relates to a composition for treating plaque formation and improving/enhancing cognitive ability comprising: (i) a macrocyclic
20 lactone in an amount effective to elevate soluble β -amyloid, generate soluble α APP and prevent β -amyloid aggregation; and (ii) a pharmaceutically effective carrier. In a preferred embodiment the composition is used to improve/enhance cognitive ability associated with Alzheimer's Disease. The macrocyclic lactone is preferably selected from the bryostatin or neristatin class compounds, particularly bryostatin-1.

25 In one embodiment of the invention the activation of PKC isoenzymes results in improved cognitive abilities. In one embodiment the improved cognitive ability is memory. In another embodiment the improved cognitive ability is learning. In another embodiment the improved cognitive ability is attention. In another embodiment PKC's isoenzymes are activated by a macrocyclic lactone (i.e.
30 bryostatin class and neristatin class). In particular, bryostatin-1 through 18 and

neristatin is used to activate the PKC isoenzyme. In a preferred embodiment bryostatin-1 is used.

In another aspect, the invention comprises a composition of a PKC isoenzyme activator administered in an amount effective to improve cognitive abilities. In a preferred embodiment the PKC isoenzyme activator is selected from macrocyclic lactones (i.e. bryostatin class and neristatin class). In a preferred embodiment the amount of PKC activator administered is in an amount effective to increase the production of sAPP. In a more preferred embodiment the amount of composition administered does not cause myalgia.

10 In a preferred embodiment the PKC isoenzymes are activated in subjects, which are suffering or have suffered from neurological diseases, strokes or hypoxia. In a more preferred embodiment the PKC isoenzyme is activated in Alzheimer's Disease subjects or models.

In another embodiment of the invention the PKC activation results in the modulation of amyloid precursor protein metabolism. Further the modulation by the PKC activation results in an increase in the alpha secretase pathway. The alpha secretase pathway results in non-toxic, non-amyloidogenic fragments related to cognitive impairment. As a result the cognitive condition of the subject improves. In another embodiment of the invention the PKC activation reduces the amyloidogenic and toxic fragments Abeta 40 and Ab42.

20 Another embodiment of the invention is a method of improving cognitive ability through the activation of PKC isoenzymes. In another embodiment of the invention the PKC activation occurs in "normal" subjects. In another embodiment of the invention the PKC activation occurs in subjects suffering from a disease, deteriorating cognitive faculties, or malfunctioning cognition. In a preferred embodiment the method is a method for treating Alzheimer's Disease.

In another embodiment of the invention the modulation of PKC is through the use of a non-tumor promoting agent resulting in improved cognitive abilities. In a preferred embodiment the PKC activator is selected from bryostatin-1 through bryostatin-18 and neristatin. In a more preferred embodiment bryostatin-1 is used.

In another embodiment bryostatin-1 is used in combination with a non-bryostatin class compound to improve cognitive ability and reduce side effects.

In another embodiment of the invention, the modulation of PKC through macrocyclic lactones (i.e. bryostatin class and neristatin class) is used *in vitro* for the testing of conditions associated with Alzheimer's Disease. The *in vitro* use may include for example, the testing of fibroblast cells, blood cells, or the monitoring of ion channel conductance in cellular models.

In a preferred embodiment of the invention the compounds and compositions are administered through oral and/or injectable forms including intravenously and intraventricularly.

The present invention therefore provides a method of treating impaired memory or a learning disorder in a subject, the method comprising administering thereto a therapeutically effective amount of one of the present compounds. The present compounds can thus be used in the therapeutic treatment of clinical conditions in which memory defects or impaired learning occur. In this way memory and learning can be improved. The condition of the subject can thereby be improved.

The compositions and methods have utility in treating clinical conditions and disorders in which impaired memory or a learning disorder occurs, either as a central feature or as an associated symptom. Examples of such conditions which the present compounds can be used to treat include Alzheimer's disease, multi-infarct dementia and the Lewy-body variant of Alzheimer's disease with or without association with Parkinson's disease; Creutzfeld-Jakob disease and Korsakow's disorder.

The compositions and methods can also be used to treat impaired memory or learning which is age-associated, is consequent upon electro-convulsive therapy or which is the result of brain damage caused, for example, by stroke, an anesthetic accident, head trauma, hypoglycemia, carbon monoxide poisoning, lithium intoxication or a vitamin deficiency.

The compounds have the added advantage of being non-tumor promoting and already being involved in phase II clinical trials.

The invention relates to a pharmaceutical composition for enhancing cognition, preventing and/or treating cognition disorders. More particularly, it relates to the pharmaceutical composition comprising macrocyclic lactones (i.e. bryostatin class and neristatin class) and their derivatives as the active ingredient for enhancing cognition, preventing and/or treating cognition disorders.

It is therefore a primary object of the invention to provide pharmaceutical compositions for enhancing cognition, preventing and/or treating cognition disorders. The pharmaceutical composition comprises macrocyclic lactones, particularly the bryostatin and neristatin class, or a pharmaceutically acceptable salt or derivative thereof, and a pharmaceutically acceptable carrier or excipient.

The pharmaceutical composition according to the invention is useful in the enhancement of cognition, prophylaxis and/or treatment of cognition disorders, wherein cognition disorders include, but are not limited to, disorders of learning acquisition, memory consolidation, and retrieval, as described herein.

The invention concerns a method for the treatment of amyloidosis associated with neurological diseases, including Alzheimer's disease by administering to a patient an effective amount of at least one agent that modulates or affects the phosphorylation of proteins in mammalian cells.

The invention also provides a method for treating Alzheimer's disease comprising administering to a patient an effective amount of a macrocyclic lactone (i.e. bryostatin class and neristatin class).

In another embodiment the bryostatin or neristatin class compounds may be used in the above methods in combination with different phorbol esters to prevent or reduce tumorigenic response in the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1(a) illustrates the effect of different PKC inhibitors and concentrations on sAPP α secretion with Bryostatin-1 showing greater efficacy at lower concentrations than controls and Benzolactam - Bryostatin (0.1 nM, solid bar) dramatically enhanced the amount of sAPP- α in the medium after 3 h incubation in a well characterized, autopsy confirmed AD cell line ($p < 0.0001$, ANOVA). The

graph units are relative to the vehicle, DMSO, alone. Bryostatin was significantly ($p < 0.01$, Tukey's post test) more potent than another PKC activator, BL, at the same (0.1 nM) concentration. Pre-treatment (rightmost bar) with staurosporin (100 nM) completely abolished the effect of bryostatin (0.1 nM). Bryostatin was also effective
5 in enhancing secretion in two control cell lines, although to a lesser extent than in the AD cell line (hatched bar);

Fig. 1(b) illustrates the effect of different concentrations of Bryostatin-1 on sAPP α secretion over a time course - Secretion is clearly near enhanced by 15 min incubation (bryostatin 0.1 nM) and near maximal at 160 incubation, remaining
10 elevated up to 3 h. Bryostatin at lower, 0.01 nM, was much slower but had about the same effect on secretion after 120 min incubation;

Fig. 1(c) illustrates the secretion of sAPP α under various experimental conditions and cells lines through a Western blot representation of sAPP- α in human fibroblasts;

15 Fig. 2 illustrates the effect of different concentrations of Bryostatin-1 on the PKC α isozyme.

Fig. 3 illustrates the amount of time required for treated rats verse controls to learn a water maze - The learning curves in the Morris Water Maze show that bryostatin (i.v.c.) improved the performance of the animals as evidenced by
20 reduction of the escape latency from early trials;

Fig. 4(a) illustrates the amount of time control rats spent swimming in the different quadrants - Both controls and treated animals show retention of preference for the target quadrant (see also figure 4(b);

25 Fig. 4(b) illustrates the amount of time treated rats spent swimming in the different quadrants.

Fig. 4(c) illustrates the different between the amount of time the treated rats spent in target quadrant compared to control rats - Treated animals showed improved retention compared to controls;

30 Fig. 5 (a) illustrates PKC translocation in human fibroblasts with bar graphs showing the ratios between the immunoreactivity (normalized by total protein content) of the membrane bound PKC (P=particulate) and the immunoreactivity

detected in the cytosolic fraction (S=soluble). PKC- α translocation was marked after 30 min incubation with 0.1 nM bryostatin (solid bar). Translocation was still present (P > S) at 180 min incubation (rightmost bar).

Fig. 5(b) illustrates other PKC isoenzymes were detected and their
5 translocation level was comparable to that observed for PKC- α

Fig. 6(a) illustrates *in vivo* testing using transgenic mice (young animals) with treatment beginning from just after weaning (3 weeks) with BL 1 mg/kg (i.p., daily) for 17 weeks. There was a significant increase in sAPP- α in the brains of the treated group compared to vehicle alone.

10 Figure 6(b) illustrates the same animals had a proportional reduction of A β 40;

Fig. 7(a) illustrates *in vivo* testing using Transgenic mice (adult animals) of approximately 6 months of age which received BL and LQ12 treatments at doses and schedules indicated in bar graphs for 7 weeks. There were small increases in
15 sAPP- α with treatments indicated by the solid bars.

Fig (7b) illustrates the small A β 40 reduction (not significant) which was observed in animals treated with BL and LQ12, both 10 mg/kg –weekly (solid bars). An unexpected (hatched bar) increase in A β 40 was observed in animals treated with BL 10 mg/kg –daily.

20 Fig. 8 illustrates sAPP α secretion in human fibroblast cells following administration of bryostatin 0.1 nM for both controls and AD cells.

Fig. 9 illustrates an immunoblot for sAPP following administration of bryostatin in AD cells.

Fig. 10 illustrates the positive effect of Bryostatin on treated mice and the
25 increase in life span compared to controls.

Fig. 11 illustrates the duration of time spent in a water test for treated animals versus non-treated animals.

Fig. 12 illustrates the decreased concentration of soluble A β -40 in treated animals versus controls.

30 Fig. 13 illustrates the decreased concentration of soluble A β -42 in treated animals versus controls.

Fig. 14 illustrates the decreased percent of plaques found in treated animal compared to controls following Thioflavin S staining.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 Memory loss and impaired learning ability are features of a range of clinical conditions. For instance, loss of memory is the most common symptom of dementia states including Alzheimer's disease. Memory defects also occur with other kinds of dementia such as multi-infarct dementia (MID), a senile dementia caused by cerebrovascular deficiency, and the Lewy-body variant of Alzheimer's disease with
10 or without association with Parkinson's disease, or Creutzfeld-Jakob disease. Loss of memory is a common feature of brain-damaged patients. Brain damage may occur, for example, after a classical stroke or as a result of an anesthetic accident, head trauma, hypoglycemia, carbon monoxide poisoning, lithium intoxication, vitamin (B1, thiamine and B12) deficiency, or excessive alcohol use or Korsakow's
15 disorder. Memory impairment may furthermore be age-associated; the ability to recall information such as names, places and words seems to decrease with increasing age. Transient memory loss may also occur in patients, suffering from a major depressive disorder, after electro-convulsive therapy (ECT). Alzheimer's disease is in fact the most important clinical entity responsible for progressive
20 dementia in ageing populations, whereas hypoxia/stroke is responsible for significant memory defects not related to neurological disorders.

 Individuals with Alzheimer's disease are characterized by progressive memory impairments, loss of language and visuospatial skills and behavior deficits (McKhann et al., 1986, Neurology, 34:939-944). The cognitive impairment of
25 individuals with Alzheimer's disease is the result of degeneration of neuronal cells located in the cerebral cortex, hippocampus, basal forebrain and other brain regions. Histologic analyses of Alzheimer's disease brains obtained at autopsy demonstrated the presence of neurofibrillary tangles (NFT) in perikarya and axons of degenerating neurons, extracellular neuritic (senile) plaques, and amyloid plaques inside and
30 around some blood vessels of affected brain regions. Neurofibrillary tangles are

abnormal filamentous structures containing fibers (about 10 nm in diameter) that are paired in a helical fashion, therefore also called paired helical filaments. Neuritic plaques are located at degenerating nerve terminals (both axonal and dendritic), and contain a core compound of amyloid protein fibers. In summary, Alzheimer's disease is characterized by certain neuropathological features including intracellular neurofibrillary tangles, primarily composed of cytoskeletal proteins, and extracellular parenchymal and cerebrovascular amyloid. Further, there are now methods in the art for distinguishing between Alzheimer's patients, normal aged people, and people suffering from other neurodegenerative diseases, such as Parkinson's, Huntington's chorea, Wernicke-Korsakoff or schizophrenia further described for instance in U.S. patent 5,580,748 and U.S. patent 6,080,582.

Alzheimer's disease is a brain disorder characterized by altered protein catabolism and characteristically presents with early memory loss. The most characteristic clinical manifestation of AD is memory loss. Memory loss occurs typically early in the course of the disease and primarily affects learning of recent information. The molecular and cellular processes that are relevant for normal associative memory storage and are affected or dysregulated in cells from AD patients are a means for treating or alleviating AD and/or improving memory. A central and potentially critical locus of convergence between memory acquisition and memory loss in AD is protein kinase C. A number of molecules and molecular events important for associative memory in animal models have been shown to be altered or defective in AD. These include, K^+ channels, calcium regulation and Protein kinase C (PKC). PKC is also involved in the processing of the Amyloid Precursor Protein (APP), a central element in AD pathophysiology. Altered protein phosphorylation has been implicated in the formation of the intracellular neurofibrillary tangles found in Alzheimer's disease. A role for protein phosphorylation in the catabolism of the amyloid precursor protein (APP), from which is derived the major component of amyloid plaques found in AD, has also been investigated. A central feature of the pathology of Alzheimer's disease is the deposition of amyloid protein within plaques.

The processing of the amyloid precursor protein (APP) determines the

production of fragments that later aggregate forming the amyloid deposits characteristic of Alzheimer's disease (AD), known as senile or AD plaques. Thus, APP processing is an early and key pathophysiological event in AD.

Three alternative APP processing pathways have been identified. The previously termed "normal" processing involves the participation of an enzyme that cleaves APP within the A β sequence at residue Lys16 (or between Lys16 and Leu17; APP770 nomenclature), resulting in non-amyloidogenic fragments: a large N-terminus ectodomain and a small 9 kDa membrane bound fragment. This enzyme, yet to be fully identified, is known as α -secretase. Two additional secretases participate in APP processing. One alternative pathway involves the cleavage of APP outside the A β domain, between Met671 and Asp672 (by β -secretase) and the participation of the endosomal-lysosomal system. An additional cleavage site occurs at the carboxyl-terminal end of the A β portion, within the plasma membrane after amino acid 39 of the A β peptide. The secretase (γ) action produces an extracellular amino acid terminal that contains the entire A β sequence and a cell-associated fragment of ~6kDa. Thus, processing by β and γ secretases generate potential amyloidogenic fragments since they contain the complete A β sequence. Several lines of evidence have shown that all alternative pathways occur in a given system and that soluble A β may be a "normal product." However, there is also evidence that the amount of circulating A β in CSF and plasma is elevated in patients carrying the "Swedish" mutation. Moreover, cultured cells transfected with this mutation or the APP₇₁₇ mutation, secrete larger amounts of A β . More recently, carriers of other APP mutations and PS1 and PS2 mutations have been shown to secrete elevated amounts of a particular form, long (42-43 amino acids) A β .

Therefore, although all alternative pathways may take place normally, an imbalance favoring amyloidogenic processing occurs in familial and perhaps sporadic AD. These enhanced amyloidogenic pathways ultimately lead to fibril and plaque formation in the brains of AD patients. Thus, intervention to favor the non-amyloidogenic, α -secretase pathway effectively shifts the balance of APP processing towards a presumably non-pathogenic process that increases the relative amount of sAPP compared with the potentially toxic A β peptides.

The PKC isoenzymes provides a critical, specific and rate limiting molecular target through which a unique correlation of biochemical, biophysical, and behavioral efficacy can be demonstrated and applied to subjects to improve cognitive ability.

5 The present inventors have studied bryostatins as activators of protein kinase (PKC). Alterations in PKC, as well alterations in calcium regulation and potassium (K^+) channels are included among alterations in fibroblasts in Alzheimer's disease (AD) patients. PKC activation has been shown to restore normal K^+ channel function, as measured by TEA-induced $[Ca^{2+}]$ elevations. Further patch-clamp data
10 substantiates the effect of PKC activators on restoration of 113pS K^+ channel activity. Thus PKC activator-based restoration of K^+ channels has been established as an approach to the investigation of AD pathophysiology, and provides a useful model for AD therapeutics. (See pending applications 09/652,656 herein incorporated in its entirety.)

15 The use of peripheral tissues from Alzheimer's disease (AD) patients and animal neuronal cells permitted the identification of a number of cellular/molecular alterations reflecting comparable processes in the AD brain and thus, of pathophysiological relevance (Baker et al., 1988; Scott, 1993; Huang, 1994; Scheuner et al., 1996; Etcheberrigaray & Alkon, 1997; Gasparini et al., 1997).
20 Alteration of potassium channel function has been identified in fibroblasts (Etcheberrigaray et al., 1993) and in blood cells (Bondy et al., 1996) obtained from AD patients. In addition, it was shown that β -amyloid, widely accepted as a major player in AD pathophysiology (Gandy & Greengard, 1994; Selkoe, 1994; Yankner, 1996), was capable of inducing an AD-like K^+ channel alteration in control
25 fibroblasts (Etcheberrigaray et al., 1994). Similar or comparable effects of β -amyloid on K^+ channels have been reported in neurons from laboratory animals (Good et al., 1996; also for a review see Fraser et al., 1997). An earlier observation of hippocampal alterations of apamin-sensitive K^+ channels in AD brains (as measured by apamin binding) provides additional support for the suggestion that K^+
30 channels may be pathophysiologically relevant in AD (Ikeda et al., 1991). Furthermore, protein kinase C (PKC) exhibits parallel changes in peripheral and

brain tissues of AD patients. The levels and/or activity of this enzyme(s) were introduced in brains and fibroblasts from AD patients (Cole et al., 1988; Van Huynh et al., 1989; Govoni et al., 1993; Wang et al., 1994). Studies using immunoblotting analyses have revealed that of the various PKC isozymes, primarily the α isoform
5 was significantly reduced in fibroblasts (Govoni et al., 1996), while both α and β isoforms are reduced in brains of AD patients (Shimohama et al., 1993; Masliah et al., 1990). These brain PKC alterations might be an early event in the disease process (Masliah et al., 1991). It is also interesting to note that PKC activation appears to favor nonamyloidogenic processing of the amyloid precursor protein,
10 APP (Buxbaum et al., 1990; Gillespie et al., 1992; Selkoe, 1994; Gandy & Greengard, 1994; Bergamashi et al., 1995; Desdouits et al., 1996; Efthimiopoulos et al., 1996). Thus, both PKC and K^+ channel alterations coexist in AD, with peripheral and brain expression in AD.

The link between PKC and K^+ channel alterations has been investigated
15 because PKC is known to regulate ion channels, including K^+ channels and that a defective PKC leads to defective K^+ channels. This is important not only for the modulation of APP, but also for the role PKC and K^+ channels play in memory establishment and recall. (e.g., see Alkon et al., 1988; Covarrubias et al., 1994; Hu et al., 1996) AD fibroblasts have been used to demonstrate both K^+ channels and
20 PKC defects (Etcheberrigaray et al., 1993; Govoni et al., 1993, 1996). Studies also show, fibroblasts with known dysfunctional K^+ channels treated with PKC activators restore channel activity as monitored by the presence/absence of TEA-induced calcium elevations. Further, assays based on tetraethylammonium chloride (TEA)-induced $[Ca^{2+}]$ elevation have been used to show functional 113pS K^+ channels that
25 are susceptible to TEA blockade (Etcheberrigaray et al., 1993, 1994; Hirashima et al., 1996). Thus, TEA-induced $[Ca^{2+}]$ elevations and K^+ channel activity observed in fibroblasts from control individuals are virtually absent in fibroblasts from AD patients (Etcheberrigaray et al., 1993; Hirashima et al., 1996). These studies demonstrate that the use of PKC activators can restore the responsiveness of AD
30 fibroblast cell lines to the TEA challenge. Further, immunoblot evidence from these studies demonstrate that this restoration is related to a preferential participation of

the α isoform.

The present inventors have also observed that activation of protein kinase C favors the α -secretase processing of the Alzheimer's disease (AD) amyloid precursor protein (APP), resulting in the generation of non-amyloidogenic soluble APP (sAPP). Consequently, the relative secretion of amyloidogenic A₁₋₄₀ and A₁₋₄₂₍₃₎ is reduced. This is particularly relevant since fibroblasts and other cells expressing APP and presenilin AD mutations secrete increased amounts of total A β and/or increased ratios of A₁₋₄₂₍₃₎/A₁₋₄₀. Interestingly, PKC defects have been found in AD brain (α and β isoforms) and in fibroblasts (α -isoform) from AD patients.

Studies have shown that other PKC activators (i.e. benzolactam) with improved selectivity for the α , β and γ isoforms enhance sAPP secretion over basal levels. The sAPP secretion in benzolactam-treated AD cells was also slightly higher compared to control benzolactam-treated fibroblasts, which only showed significant increases of sAPP secretion after treatment with 10 μ M BL. It was further reported that staurosporine (a PKC inhibitor) eliminated the effects of benzolactam in both control and AD fibroblasts while related compounds also cause a ~3-fold sAPP secretion in PC12 cells. The present inventors have found that the use of bryostatin as a PKC activators to favor non-amyloidogenic APP processing is of particular therapeutic value since it is non-tumor promoting and already in stage II clinical trials.

Memories are thought to be a result of lasting synaptic modification in the brain structures related to information processing. Synapses are considered a critical site at final targets through which memory-related events realize their functional expression, whether the events involve changed gene expression and protein translation, altered kinase activities, or modified signaling cascades. A few proteins have been implicated in associative memory including Ca²⁺/calmodulin II kinases, protein kinase C, calyculin, a 22-kDa learning-associated Ca²⁺ binding protein, and type II ryanodine receptors. The modulation of PKC through the administration of macrocyclic lactones provides a mechanism to effect synaptic modification.

The area of memory and learning impairment is rich in animal models that are able to demonstrate different features of memory and learning processes. (See,

for example, Hollister, L. E., 1990, *Pharmacopsychiat.*, 23, (Suppl II) 33-36). The available animal models of memory loss and impaired learning involve measuring the ability of animals to remember a discrete event. These tests include the Morris Water Maze and the passive avoidance procedure. In the Morris Water Maze, animals are allowed to swim in a tank divided into four quadrants, only one of which has a safety platform beneath the water. The platform is removed and the animals are tested for how long they search the correct quadrant verse the incorrect quadrants. In the passive avoidance procedure the animal remembers the distinctive environment in which a mild electric shock is delivered and avoids it on a second occasion. A variant of the passive avoidance procedure makes use of a rodent's preference for dark enclosed environments over light open ones. Further discussion can be found in Crawley, J. N., 1981, *Pharmacol. Biochem. Behav.*, 15, 695-699; Costall, B. et al, 1987, *Neuropharmacol.*, 26, 195-200; Costall, B. et al, 1989, *Pharmacol. Biochem. Behav.*, 32, 777-785; Barnes, J. M. et al, 1989, *Br. J. Pharmacol.*, 98 (Suppl) 693P; Barnes, J. M. et al, 1990, *Pharmacol. Biochem. Behav.*, 35, 955-962.

The use of the word, "normal" is meant to include individuals who have not been diagnosis with or currently display diminished or otherwise impaired cognitive function. The different cognitive abilities may be tested and evaluated through known means well established in the art, including but not limited to tests from basic motor-spatial skills to more complex memory recall testing. Non-limiting examples of tests used for cognitive ability for non-primates include the Morris Water Maze, Radial Maze, T Maze, Eye Blink Conditioning, Delayed Recall, and Cued Recall while for primate subjects test may include Eye Blink, Delayed Recall, Cued Recall, Face Recognition, Minimental, and ADAS-Cog. Many of these tests are typically used in the mental state assessment for patients suffering from AD. Similarly, the evaluation for animal models for similar purposes with well describe in the literature.

Of particular interest are macrocyclic lactones (i.e. bryostatin class and neristatin class) that act to stimulate PKC. Of the bryostatin class compounds, bryostatin-1 has been shown to activate PKC and proven to be devoid of tumor

promotion activity. Bryostatin-1, as a PKC activator, is also particularly useful since the dose response curve of bryostatin-1 is biphasic. Additionally, bryostatin-1 demonstrates differential regulation of PKC isozymes, including PKC α , PKC δ , and PKC ϵ . Bryostatin-1 has undergone toxicity and safety studies in animals and humans and is actively being investigated as an anti-cancer agent. Bryostatin-1's use in the studies has determined that the main adverse reaction in humans is myalgia, limiting the maximum dose to 40 mg/m². The present invention has utilized concentrations of 0.1 nM of bryostatin-1 to cause a dramatic increase of sAPP secretion. Bryostatin-1 has been compared to a vehicle alone and to another PKC activator, benzolactam (BL), used at a concentration 10,000 times higher. Also bryostatin used at 0.01 nM still proved effective to increase sAPP secretion. (See, Figure 1(a)). PKC translocation shows that a measure of activation is maximal at 30 min, followed by a partial decline, which remains higher than basal translocation levels up to six hours. (see Figures 1(b), 2, 8, and 9). The use of the PKC inhibitor staurosporin completely prevents the effect of bryostatin on sAPP secretion. The data further demonstrates that PKC activation mediates the effect of the bryostatin on sAPP secretion. (see Figures 1 and 2)

Macrocyclic lactones, and particularly bryostatin-1 is described in U.S. Patent 4,560,774. Macrocyclic lactones and their derivatives are described elsewhere in the art for instance in U.S. Patent 6,187,568, U.S. Patent 6,043,270, U.S. Patent 5,393,897, U.S. Patent 5,072,004, U.S. Patent 5,196,447, U.S. Patent 4,833,257, and U.S. Patent 4,611,066. The above patents describe various compounds and various uses for macrocyclic lactones including their use as an anti-inflammatory or anti-tumor agent. Other discussions regarding bryostatin class compounds can be found in: Differential Regulation of Protein Kinase C Isozymes by Bryostatin 1 and Phorbol 12-Myristate 13-Acetate in NIH 3T3 Fibroblasts, Szallasi et al., Journal of Biological Chemistry, Vol. 269, No. 3, pp. 2118-24 (1994); Preclinical Pharmacology of the Natural Product Anticancer Agent Bryostatin 1, an Activator of Protein Kinase C, Zhang et al., Cancer Research 56, 802-808 (1996); Bryostatin 1, an activator of protein kinase C, inhibits tumor promotion by phorbol esters in SENCAR mouse skin, Hennings et al., Carcinogenesis vol. 8, no. 9, pp

1343-46 (1987); Phase II Trial of Bryostatin 1 in Patients with Relapse Low-Grade Non-Hodgkin's Lymphoma and Chronic Lymphocytic Leukemia, Varterasian et al., Clinical Cancer Research, Vol. 6, pp. 825-28 (2000); and Review Article: Chemistry and Clinical Biology of the Bryostatins, Mutter et al., Bioorganic & Medicinal
5 Chemistry 8, pp. 1841-1860 (2000).

Macrocyclic lactones, including the bryostatin class, represent known compounds, originally derived from *Bugula neritina* L. While multiple uses for macrocyclic lactones, particularly the bryostatin class are known, the relationship between macrocyclic lactones and cognition enhancement was previously unknown.

10 The examples of the compounds that may be used in the present invention include macrocyclic lactones (i.e. bryostatin class and neristatin class compounds). While specific embodiments of these compounds are described in the examples and detailed description, it should be understood that the compounds disclosed in the references and derivatives thereof could also be used for the present compositions
15 and methods.

As will also be appreciated by one of ordinary skill in the art, macrocyclic lactone compounds and their derivatives, particularly the bryostatin class, are amenable to combinatorial synthetic techniques and thus libraries of the compounds can be generated to optimize pharmacological parameters, including, but not limited
20 to efficacy and safety of the compositions. Additionally, these libraries can be assayed to determine those members that preferably modulate α -secretase and/or PKC.

Combinatorial libraries high throughput screening of natural products and fermentation broths has resulted in the discovery of several new drugs. At present,
25 generation and screening of chemical diversity is being utilized extensively as a major technique for the discovery of lead compounds, and this is certainly a major fundamental advance in the area of drug discovery. Additionally, even after a "lead" compound has been identified, combinatorial techniques provide for a valuable tool for the optimization of desired biological activity. As will be appreciated, the
30 subject reactions readily lend themselves to the creation of combinatorial libraries of compounds for the screening of pharmaceutical, or other biological or medically-

related activity or material-related qualities. A combinatorial library for the purposes of the present invention is a mixture of chemically related compounds, which may be screened together for a desired property; said libraries may be in solution or covalently linked to a solid support. The preparation of many related compounds in a single reaction greatly reduces and simplifies the number of screening processes that need to be carried out. Screening for the appropriate biological property may be done by conventional methods. Thus, the present invention also provides methods for determining the ability of one or more inventive compounds to bind to effectively modulate α -secretase and/or PKC.

10 A variety of techniques are available in the art for generating combinatorial libraries described below, but it will be understood that the present invention is not intended to be limited by the foregoing examples and descriptions. See, for example, Blondelle et al. (1995) Trends Anal. Chem. 14:83; the Affymax U.S. Patents 5,359,115 and 5,362,899; the Ellman U.S. Patent 5,288,514; the Still et al. PCT publication WO 94/08051; Chen et al. (1994) JACS 116:2661; Kerr et al. (1993) JACS 115:252; PCT publications W092/10092, W093/09668 and W091/07087; and the Lemer et al. PCT publication W093/20242). Accordingly, a variety of libraries on the order of about 16 to 1,000,000 or more diversomers can be synthesized and screened for a particular activity or property.

20 The present compounds can be administered by a variety of routes and in a variety of dosage forms including those for oral, rectal, parenteral (such as subcutaneous, intramuscular and intravenous), epidural, intrathecal, intra-articular, topical and buccal administration. The dose range for adult human beings will depend on a number of factors including the age, weight and condition of the patient and the administration route.

For oral administration, fine powders or granules containing diluting, dispersing and/or surface-active agents may be presented in a draught, in water or a syrup, in capsules or sachets in the dry state, in a non-aqueous suspension wherein suspending agents may be included, or in a suspension in water or a syrup. Where desirable or necessary, flavouring, preserving, suspending, thickening or emulsifying agents can be included.

Other compounds which may be included by admixture are, for example, medically inert ingredients, e.g. solid and liquid diluent, such as lactose, dextrose, saccharose, cellulose, starch or calcium phosphate for tablets or capsules, olive oil or ethyl oleate for soft capsules and water or vegetable oil for suspensions or emulsions; lubricating agents such as silica, talc, stearic acid, magnesium or calcium stearate and/or polyethylene glycols; gelling agents such as colloidal clays; thickening agents such as gum tragacanth or sodium alginate, binding agents such as starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinylpyrrolidone; disintegrating agents such as starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuff; sweeteners; wetting agents such as lecithin, polysorbates or laurylsulphates; and other therapeutically acceptable accessory ingredients, such as humectants, preservatives, buffers and antioxidants, which are known additives for such formulations.

Liquid dispersions for oral administration may be syrups, emulsions or suspensions. The syrups may contain as carrier, for example, saccharose or saccharose with glycerol and/or mannitol and/or sorbitol. In particular a syrup for diabetic patients can contain as carriers only products, for example sorbitol, which do not metabolize to glucose or which metabolize only a very small amount to glucose. The suspensions and the emulsions may contain a carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose or polyvinyl alcohol.

Suspensions or solutions for intramuscular injection may contain, together with the active compound, a pharmaceutically acceptable carrier such as sterile water, olive oil, ethyl oleate, glycols such as propylene glycol and, if desired, a suitable amount of lidocaine hydrochloride. Solutions for intravenous injection or infusion may contain a carrier, for example, sterile water that is generally Water for Injection. Preferably, however, they may take the form of a sterile, aqueous, isotonic saline solution. Alternatively, the present compounds may be encapsulated within liposomes. The present compounds may also utilize other known active agent delivery systems.

The present compounds may also be administered in pure form unassociated with other additives, in which case a capsule, sachet or tablet is the preferred dosage form.

Tablets and other forms of presentation provided in discrete units
5 conveniently contain a daily dose, or an appropriate fraction thereof, of one of the present compounds. For example, units may contain from 5 mg to 500 mg, but more usually from 10 mg to 250 mg, of one of the present compounds.

It will be appreciated that the pharmacological activity of the compositions of the invention can be demonstrated using standard pharmacological models that
10 are known in the art. Furthermore, it will be appreciated that the inventive compositions can be incorporated or encapsulated in a suitable polymer matrix or membrane for site-specific delivery, or can be functionalized with specific targeting agents capable of effecting site specific delivery. These techniques, as well as other drug delivery techniques are well known in the art.

15 In summary, activation of PKC effects memory acquisition as well as facilitate the non-amyloidogenic, α -secretase, processing of APP. A non-tumor promoter PKC activator, bryostatin 1, dramatically enhanced the secretion of the α -secretase product, sAPP α , in fibroblasts from AD patients. The effect was prominent at sub-nanomolar concentrations of bryostatin. Bryostatin, injected
20 intraventricularly, also enhanced the performance of rats subjected to the Morris Water Maze paradigm. Recent *in vivo* studies have shown that benzolactam, a PKC activator previously shown to reverse K⁺ channels defects and to enhance sAPP α in AD cells, significantly increased the amount of sAPP α and reduced A β 40 in the brains of transgenic mice carrying the London V717I APP mutation. These results
25 demonstrate that PKC (and its activation) may a tool for treatment or alleviation of symptoms related to AD and memory loss. Bryostatin 1 is of particular interest as it is not only more potent but is devoid of tumor promoting activity and is already undergoing clinical studies for cancer treatment in humans. The below experiments provide evidence that bryostatin 1 dramatically and potently enhances the α -
30 processing of APP (generating increased amounts of sAPP α) and significantly improves rats' performance in the Morris Water Maze task. The experiments also

provide evidence that another PKC activator, benzolactam, causes a significant increase in sAPP α and reduction of A β 40 *in vivo*.

All books, articles, or patents references herein are incorporated by reference to the extent not inconsistent with the present disclosure. The present invention will
5 now be described by way of examples, which are meant to illustrate, but not limit, the scope of the invention.

Example I:

Cell Culture

Cultured skin fibroblasts were obtained from the Coriell Cell Repositories
10 and grown using the general guidelines established for their culture with slight modifications (Cristofalo & Carptentier, 1988; Hirashima et al., 1996). The culture medium in which cells were grown was Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (Biofluids, Inc.). Fibroblasts from control cell lines (AC), cases AG07141 and AG06241, and a familial AD (FAD)
15 case (AG06848) were utilized.

PKC Activators

The different tissue distributions, the apparently distinctive roles of different isozymes, and the differential involvement in pathology make it important to use pharmacological tools that are capable of preferentially targeting specific isozymes
20 (Kozikowski et al., 1997; Hofmann, 1997). Recent research in the medicinal chemistry field has resulted in the development of several PKC activators, for instance different benzolactams and pyrrolidinones. However, the currently studied bryostatin PKC activator not only has the benefit of providing isospecific activity, but also does not suffer from the set back of the previously used PKC activator, such
25 as being tumor promoting. The bryostatin competes for the regulatory domain of PKC and engages in very specific hydrogen bond interactions within this site. Additional information on the organic chemistry and molecular modeling of this compound can be found throughout the literature.

Treatment

Cells grown to confluence in 6 cm Petri dishes for 5-7 days. On the day of the experiment, medium was replaced with DMEM without serum and left undisturbed for 2h. Upon completion of the 2 hour serum deprivation, treatment was achieved by direct application to the medium of Bryo, BL and DMSO at the appropriate concentrations. (0.1 and 0.01 nM for bryostatin; 0.1 nM, 0.1 μ M, and 1 μ M BL). DMSO was less than 1% in all cases. In most cases, medium was collected and processed after 3 hours of treatment for sAPP secretion. Other time points were also used to establish a time course of secretion.

Immunoblot Assay for PKC Translocation

Immunoblot experiments were conducted using well-established procedures (Dunbar, 1994). Cells were grown to confluency (~90%) in 6 cm Petri dishes. Levels of isozyme in response to treatment with 0.1 nM bryostatin-1 for 5, 30, 60, and 120 minutes was quantified using procedures slightly modified from that established by Racchi et al., (1994). Fibroblasts were washed twice with ice-cold PBS, scraped in PBS, and collected by low-speed centrifugation. The pellets were re-suspended in the following homogenization buffer: 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2mM EGTA, 5 mM DTT, 0.32 M sucrose, and protease inhibitor cocktail (Sigma). Homogenates were obtained by sonication, and centrifuged at ~12,000g for 20 minutes, and the supernatants were used as the cytosolic fraction. The pellets were homogenized in the same buffer containing 1.0% Triton X-100, incubated in ice for 45 minutes, and centrifuged at ~12,000g for 20 minutes. The supernatant from this batch was used as the membranous fraction. After protein determination, 20 μ g of protein were diluted in 2 X electrophoresis sample buffer (Novex), boiled for 5 minutes, run on 10% acrylamide gel, and transferred electrophoretically to a PVDF membrane. The membrane was saturated with 5% milk blocker by incubating it at room temperature for an hour. The primary antibody for PKC isoform (Transduction Laboratories) was diluted (1:1000) in blocking solution and incubated with the membrane overnight at 4°C. After incubation with the secondary antibody, alkaline phosphatase antimouse IgG (Vector Laboratories), the membrane was developed using a chemoluminescent

substrate (Vector Laboratories) per the manufacturer's instructions. The band intensities were quantified by densitometry using a BioRad GS-800 calibrated scanning densitometer and Multianalyst software (BioRad).

sAPP - Determinations/Measurements of sAPP α

5 The concentration of secreted APP was measured using conventional immunoblotting techniques, with minor modifications the protocol. Precipitated protein extracts from each dish/treatment were loaded to freshly prepared 10% acrylamide Tris-HCl minigels and separated by SDPAGE. The volume of sample loaded was corrected for total cell protein per dish. Proteins were then
10 electrophoretically transferred to PVDF membranes. Membranes were saturated with 5% non-fat dry milk to block non-specific binding. Blocked membranes were incubated overnight at 4 °C with the commercially available antibody 6E10 (1:500), which recognizes sAPP-alpha in the conditioned medium (SENTEK). After washing, the membranes were incubated at room temperature with horseradish
15 peroxidase conjugated anti-mouse IgG secondary antibody (Jackson's Laboratories). The signal was then detected using enhanced chemiluminescence followed by exposure of Hyperfilm ECL (Amersham). The band intensities were quantified by densitometry using a BioRad GS-800 calibrated scanning densitometer and Multianalyst software (BioRad).

20 As shown in Fig. 8 and 9, Bryostatin-1 elicits a powerful response, demonstrating the activation of PKC. It should be noted the activation of PKC is easily detectable 30 minutes after delivery, following a dose of only 0.1 nM of bryostatin-1.

 It is also interesting to consider the data in relation to APP metabolism and
25 the effects of its sub-products. Studies have demonstrated that PKC activation increases the amount of ratio of non-amyloidogenic (soluble APP, presumably product of the secretase) vs. amyloidogenic (A β 1-40 and/or A β 1-42) secreted fragments (Buxbaum et al., 1990; Gillespie et al., 1992; Selkoe, 1994). Without wishing to be held to this theory, one could speculate that AD cells with low PKC
30 would have an impaired secretion of sAPP and/or have increased proportion of

amyloidogenic fragments. Indeed, there is evidence that some AD cell lines exhibit both defective PKC and impaired sAPP secretion (Bergamaschi et al., 1995; Govoni et al., 1996). In addition, β -amyloid has been shown to induce an AD-like K^+ channel defect in fibroblasts (Etcheberrigaray et al., 1994) and to block K^+ currents in cultured neurons (Good et al., 1996). Therefore, we suggest a mechanistic link such that an isozyme-specific PKC defect may lead to abnormal APP processing that, among other possible deleterious effects, alters K^+ channel function. Recent preliminary data also suggest that, perhaps in a vicious cyclical manner, β -amyloid in turn causes reductions of PKC (Favit et al., 1997).

In summary, the data suggest that the strategy to up-regulate PKC function targeting specific isozymes increases sAPP production. These studies and such a fibroblast model could be expanded and used as tools to monitor the effect of compounds (bryostatin, for example) that alter potential underlying pathological processes. Further, one of ordinary skill in the art would know how to further test these samples through Ca^{2+} imaging and electrophysiology. Such compounds could then be used as bases for rational design of pharmacological agents for this disorder.

Example II: Behavioral Studies

The Morris Water Maze paradigm (48) was used to study the effects of bryostatin 1 in learning and memory. Wistar albino rats (n=20) weighing between 220-250 g were housed for one week with free access to food and water. Stainless steel cannulas were placed bilaterally in each rat, following previously described procedures (49). All animals had a one-week recovery period prior to any further experimentation. Subsequently, animals were assigned randomly to experimental and control groups. At least 24 h prior to treatment and training, all animals were pre-exposed to the MWM experimental situation by placing them in the water and allowed to swim for 120 s. The training followed standard procedures (49) and consisted of two trials per day for 4 consecutive days. Treated animals received (i.c.v.) 1 μ l/site of a 2 μ M solution of bryostatin 1 approximately 30 min prior to training trials 1 and 5. The control group received the same volume of vehicle alone,

on identical schedule. On the fifth day, the platform was removed and the retention test was conducted. Animals' movements and escape latencies were recorded with an automatic tracking system. Learning was measured as the reduction of escape latency from trial to trial, which was significantly lower in the treated animals.

5 Acquisition of memory was measured as time spent in the relevant quadrant (5th day). Memory or retention was significantly enhanced in treated animals, compared to sham injection animals (see Figures 3 through 4(a)-4(c)). The rats treated with bryostatin-1 showed improved cognition over control rats within 2 days of treatment (see Figure 3). Bryostatin-1 is capable of being used at concentrations to improve

10 cognition that are 300 to 300,000 times lower than the concentration used to treat tumors. The above example further shows that cognitive ability can be improved in non-diseased subjects as compared to other non-diseased subjects through the administration of bryostatin-1.

Because of the previously conducted safety, toxicology and phase II clinical

15 studies for cancer, one can conclude that the use of PKC activators, particularly bryostatin-1, would be viewed as safe and that phase II studies for AD treatment/cognitive enhancement could be expedited. Furthermore, bryostatin-1's lipophilic nature provides increased blood brain barrier transport. The present invention would allow for intravenous, oral, intraventricular, and other known

20 methods of administration.

Test of sAPP secretion experiments, PKC activation experiments, and animal behavior experiments have shown that increases in sAPP secretion follow increased PKC activation and result in improved cognition in animal behavior studies.

Example III: Transgenic animals and *in vivo* studies

25 Transgenic mice carrying the V717I mutation were treated with BL (1mg/kg, i.p.; daily) from ~ 3 weeks of age (after weaning) for 17 weeks (n=4). The control group (n=4) received vehicle alone (Tween 20 1%, DMSO 25%, 74% PBS). Another experimental group consisted of 5-6 months old animals treated for 7 weeks. Subgroups of these animals were treated with BL 1mg/kg, daily (n=5); BL 10

30 mg/kg, daily (n=3; due to two deaths); BL 10 mg/kg, weekly (n=4; one death),

LQ12 10 mg/kg, daily (n=5); and LQ12 10 mg/kg, weekly (n=5). Five additional animals received vehicle alone for the same period. After completion of the treatment, animals were euthanized according to K.U.L. (Belgium) guidelines. Brains were removed and prepared for biochemical analyses of APP species.

5 **Biochemical analysis of APP processing in brain of APP tg mice**

Immunoblot analysis. The biochemical analysis of intermediates of APP metabolism has been described elsewhere by Dewachter et al. (Aging increased amyloid peptide and caused amyloid plaques in brain of old APP/V717I transgenic mice by a different mechanism than mutant presenilin1. J Neurosci. 2000; 20:6452-8.). Briefly, brains were homogenized in 6.5 vol. of ice-cold buffer containing 20 mM Tris-HCl, pH 8.5, and a mixture of proteinase inhibitors (Roche, Darmstadt, Germany). After centrifugation at $135,000 \times g$ at 4°C for 1 hr, the supernatant was centrifuged again for 2 hr at $200,000 \times g$ before analysis of soluble amyloid peptides by specified ELISA. The pellets from the first centrifugation were resuspended in 15 TBS containing 2% Triton X-100, 2% Nonidet P40 and proteinase inhibitors and centrifuged at $100,000 \times g$ at 4°C for 1hr. This protein fraction was used for analysis of membrane-bound APP. Western blotting of membrane bound APP was performed on this protein fraction containing membrane-bound proteins, with monoclonal antibody 8E5. Total secreted APP and α -secretase cleaved secreted 20 APP- α were detected by Western blotting analysis on the supernatant of the first centrifugation, with monoclonal antibody 8E5 and monoclonal antibody JRF14, respectively. Proteins were denatured and reduced in sample buffer containing a final concentration of 2% SDS, 1% 2-ME and separated on 8% TRIS Glycine gels (Novex, San Diego, CA). After incubation with appropriate secondary antibodies, all 25 Western blots were developed with the ECL detection system and photographically recorded. Application of a series of diluted sample allowed quantitation. Densitometric scanning of films and normalization were performed using a flatbed optical density scanner and dedicated software for analysis and measurement (Image Master; Pharmacia, Uppsala, Sweden).

ELISA of amyloid peptides. Protein extracts were applied on reversed-phase columns (C18-Sep-pack cartridges; Waters Corporation, Milford, MA) and washed with increasing concentrations of acetonitrile (5, 25, and 50%) containing 0.1% trifluoroacetic acid. The last fraction contained the amyloid peptides and was dried
5 *in vacuo* overnight and dissolved for measurements in ELISA. Sandwich ELISA for human A β 40 and A β 42 peptides was performed using the capture antiserum JRF/cA β 40/10 and 21F12, respectively, and they were developed with monoclonal antibodies JRFcA β tot/14hrpo and 3D6, respectively (Vanderstichele H, Van Kerschaver E, Hese C, Davidsson P, Buyse MA, Andreassen N, Minthon L, Wallin
10 A, Blennow K, Vanmechelen E. Standardization of measurements of beta-amyloid (1-42) in cerebrospinal fluid and plasma. *Amyloid* 2000; 7: 245-258).

Standard general health assessment and open field were conducted in all animals prior to the biochemical assessments. In addition, a semi-quantitative *ad hoc* score was devised to measure abdominal contractions that followed the injections (+
15 = weak, ≤ 2 min; ++: strong, ≥ 2 min; +++: very strong, ≥ 2 min).

Example IV: Transgenic Animals and *in vivo* Studies Using Bryostatin

A second transgenic study using similar procedures/testing and protocol was performed using double transgenic mice carrying the V717I mutation and a Presenilin-1 (PS1) mutation, which causes accelerated amyloid formation, with the
20 following major differences. Approximately 40 mice including both treated and controls were utilized. Treatment began at approximately 3 weeks of age and consisted of treatments with 40 μ g/k.g. i.p. three times a week using Bryostatin-1. Controls were given vehicle alone. The treatment continued for approximately seven months before the morbidity rate of the non-treated animals necessitated termination
25 of the experiment (See, Fig. 10). While behavioural differences between the treated and non-treated animals were not significant using water testing (See, Fig. 11), treated animals demonstrated decreases in soluble A β -40 (See, Fig. 12) and soluble A β -42 (See, Fig. 13). Additionally, the treated mice demonstrated an overall lower amount of total APP as show in Figure 14 where Thioflavin S staining shows a
30 decrease in percent plaque load compared to controls.

Discussion of Above Experiments

sAPP α secretion: After three hours of treatment of AD cell line AG06848 with 0.1 nM bryostatin, there was a dramatic increase in the secretion of sAPP α compared to all other conditions, overall ANOVA, $p < 0.0001$ (Fig. 1(a), solid bar). This effect was also significantly higher than another PKC activator, BL, used at the same (0.1 nM) concentration ($p < 0.01$, Tukey's post-test). BL 0.1 nM, had no real impact on secretion and it was no different than DMSO alone. Pre-treatment with 100 nM of staurosporin, a PKC blocker, abolished the effects of 0.1 nM bryostatin (Fig. 1A, rightmost bar). Two cell lines were also used from age-matched controls. In these cell lines (pooled), bryostatin (0.1 nM) also significantly (compared to DMSO alone, $p < 0.05$, Tukey's) enhanced the secretion of sAPP α , but to a significant lesser extent than in the AD cell line (Fig. 1A, hatched bar; $p < 0.05$, Tukey's). A time-course experiment (Fig. 1(b), inset) showed a marked increase in sAPP α secretion after 15 min incubation with 0.1 nM bryostatin. Progressive and proportional increases were observed at 30 and 60 min. Incubation periods, 2 and 3 h did not substantially differ from 60 min incubation in terms of the amount of APP α secreted. The lower concentration of bryostatin, 0.01 nM, produced a robust enhancement of APP α secretion only after 60 min of incubation. The effect of the low concentration (0.01 nM), however, was undistinguishable from the higher (0.1nM bryostatin) at 2 and 3 h of incubation (Fig. 1(b)). Representative immunoblots illustrating the secretion of sAPP α under various experimental conditions and cells lines are depicted in Fig. 1(c).

PKC translocation: Levels of cytosolic and membrane-bound of the α isoenzyme were determined after incubation with bryostatin (various time points) at 0.1 and 0.01 nM. There was a relative increase (compared to DMSO alone) in the membrane bound component of the PKC α -isoenzyme, measured as the ratio particulate/soluble (P/S) immunoreactivity. The increase was most consistent and significantly different than DMSO alone ($p=0.411$; t-test, two-tailed) after 30 min incubation. The P/S ratio progressively declined, but remained higher (albeit not statistically significant) than DMSO alone even after 180 min of incubation (Fig. 5a). Short-term incubation (5 min) did not induce a consistent or significantly

different translocation than DMSO alone (not shown). The effect of 0.01 nM bryostatin was much less marked and slow, with a maximum P/S ratio value at 120 min incubation. Levels of translocation of other PKC isoenzymes were assessed at 30 min incubation with 0.1 nM bryostatin. Clear immunoreactivity was detected
5 (both membrane-bound and cytosolic) with specific antibodies for ϵ , β and δ isoenzymes. The ratio S/P was higher in all cases than DMSO alone and comparable to the levels of PKC- α (Fig. 5b).

Behavior (MWM): The learning curve of the group receiving bryostatin was significantly faster than the control group. Escape latencies were clearly reduced
10 from early trials and lower than the control group from trial 3. The quadrant preference test showed retention in both groups, but was significantly enhanced for the bryostatin treated group, compared to controls. Fig. 3-4(c) summarizes these results.

Transgenic animals: The transgenic animals treated with BL from 3 weeks of age
15 for 17 weeks showed a significant increase in sAPP- α and a concomitant and proportional reduction in A β 40 (Fig. 6 (a)-(b)). There were no differences in the amount of A β 42, APP membrane-bound and total secreted sAPP (sAPP α + sAPP β). Animals showed no differences in general health and weight gain was similar in both groups. Injections caused variable abdominal contractions (reversible) with
20 similar frequency in both groups. The intensity was somewhat elevated in the BL-treated group (data not shown). In addition, BL treated animals showed an increase in open field test scores, without reaching statistical significance (not shown).

The animals treated later in life (6 months of age) and for a shorter period (~7 weeks) did not show any dramatic changes in terms of APP species. The general
25 trend (small changes), however, was in the same direction as described for the longer-term treatment (previous section). There was slight increase in sAPP α in animals treated with BL 10 mg/kg (daily and weekly) and also in animals treated with LQ12 10 mg/kg, daily (Fig 7(b), solid bars). BL 1mg/kg (daily) and LQ12 10 mg/kg (weekly) had no effect (fig 5A, pattern bars). A slight decrease in A β 40 was
30 observed in animals treated with BL (n=5) and LQ12 (n=5), both 10 mg/kg, weekly

(Fig. 7(b), solid bars). There was no noticeable significant change in A β 42 with the treatments. Similarly, there was no significant change in total soluble APP and membrane-bound APP. Abdominal contractions and flaccidity of the hind legs were also observed in the older animals upon injections (reversible). They seemed related to dose but no clear systems were in place for more accurate assessment. General health and weights were also normal. A few (2-3) animals died (7.8 % of the total) during the course of the experiment of causes that do not appear related to the treatment. There were no differences in the open field test (not shown).

However, treatment with Bryostatin-1 showed a noticeable change in both A β -40, A β -42, and total APP. (See, Fig. 12-14). Additionally, animals treated with Bryostatin-1 demonstrated a greater life percentage over time. (See, Fig. 10).

These results demonstrate PKC's role in AD pathophysiology. These results further demonstrate that there is a common APP pool. Therefore, increase of one enzymatic pathway results in less substrate for an alternative enzyme. In this case, a reduction of an amyloidogenic and toxic fragment (A β 40) is achieved by increasing the non-pathogenic α -secretase processing of APP. The fact that the total secreted APP (α + β products) is not different between treated and untreated animals, is consistent with- and confirms the interpretation. It is also apparent that the increase in sAPP α is not the result of elevated total APP (or increased expression), since membrane bound APP is similar in both groups.

It is important to note that the most marked "beneficial" effect was observed in animals that had begun treatment early in life and for a longer period. This suggests that preventing long-term effects of toxic fragments should be an important goal of therapies. Intervention later in life and later in the course of the disease process (even without clinical manifestations), as suggested by the results obtained in older animals, would have much less impact in preventing damage by toxic fragments. It is also important to mention that this particular transgenic model first causes biochemical alterations, followed by cognitive deficits, and then, much later, amyloid deposition and plaques. In agreement with *in vitro* studies this sequence shows that amyloid species can be deleterious (presumably in the soluble form) before any significant deposition has taken place.

The results showing an improvement performance of normal rats in the MWM task after bryostatin administration (i.c.v.) demonstrate that PKC activation can cause cognitive enhancement as an added therapeutic effect. Additionally, secreted APP may by itself improve memory in normal and amnesic mice. These
5 experiments and models demonstrate the PKC regulation, particularly through bryostatin-1 can result in an increase in sAPP and/or an improvement in memory. They also demonstrate that a regime which includes a PKC activator can be used to prevent build up of toxic fragments and prevent memory decline.

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WHAT IS CLAIMED IS:

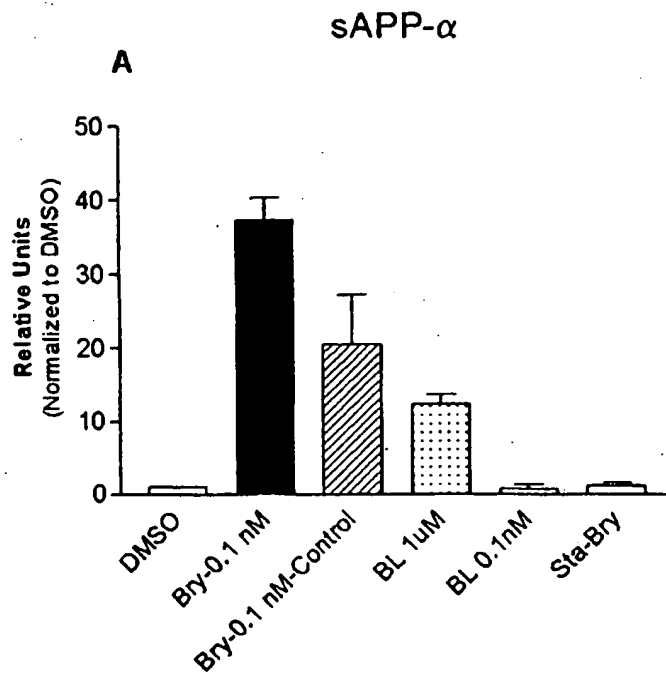
1. A method for enhancing cognitive ability in a human or animal, comprising administering to said human or animal a PKC activator in an amount effective for enhancing cognitive ability in a pharmaceutically acceptable carrier.
- 5 2. The method of claim 1 wherein the PKC activator selectively activates PKC α , PKC δ , and PKC ϵ .
3. The method of claim 1, wherein the PKC activator is a macrocyclic lactone, a benzolactam, a pyrrolidinone or a combination thereof.
4. The method of claim 3, wherein the macrocyclic lactone is a bryostatin class or
10 neristatin class compound.
5. The method of claim 3 wherein the PKC activator is bryostatin-1 through bryostatin 18 or neristatin-1.
6. The method of claim 1, wherein the cognitive ability enhanced is learning, memory, or attention.
- 15 7. The method of claim 5, wherein the animal is a primate.
8. The method of claim 5, wherein the animal is a non-primate.
9. The method of claim 1, wherein the amount of PKC activator administered is in an amount effective to treat cognitive impairment of a neurological disease or disorder.
- 20 10. The method of claim 9, wherein the neurological disease is Alzheimer's Disease, multi-infarct dementia, the Lewy-body variant of Alzheimer's Disease with or without association with Parkinson's disease; Creutzfeld-Jakob disease, Korsakow's disorder, or attention deficit hyperactivity disorder.
11. The method of claim 9, wherein the disorder is -associated with age, electro-
25 convulsive therapy or brain damage.

12. The method of claim 11, wherein the brain damage was caused by stroke, an anesthetic accident, head trauma, hypoglycemia, carbon monoxide poisoning, lithium intoxication or a vitamin deficiency.
13. The method of claim 1, wherein the PKC activator is administered in an amount
5 effected to cause an increase in sAPP.
14. A method for altering cellular modulation of ion channels comprising administering a PKC activator in an amount effective for altering cellular modulation of ion channels and a pharmaceutically acceptable carrier.
15. The method of claim 14 wherein, said modulation is *in vivo* or *in vitro*
10 modulation.
16. The method of claim 15, wherein said ion channel is a K^+ or Ca^{++} channel.
17. A method for treating neurotumors comprising administering macrocyclic lactone in an amount effective to treat said neurotumors and a pharmaceutically acceptable carrier.
- 15 18. The method of claim 17 wherein, the macrocyclic lactone is a bryostatin class or neristatin class compound.
19. A method for modulating sAPP comprising administering a macrocyclic lactone in an amount effective to modulate sAPP and a pharmaceutically acceptable carrier.
- 20 20. The method of claim 19 wherein, the macrocyclic lactone activates PKC.
21. The method of claim 19 wherein, the macrocyclic lactone is a bryostatin class or a neristatin class compound.
22. The method of claim 19 wherein, the macrocyclic lactone is bryostatin-1 through bryostatin 18, or neristatin-1.
- 25 23. A method for modulating α -secretase comprising administering a macrocyclic lactone in an amount effective to modulate α -secretase and a pharmaceutically suitable carrier.

24. The method of claim 23 wherein, the macrocyclic lactone is a bryostatin class or a neristatin class compound.
25. The method of claim 23 wherein, the macrocyclic lactone is bryostatin-1 through bryostatin 18, or neristatin-1.
- 5 26. The method of claim 23 wherein, the macrocyclic lactone is administered *in vivo* or *in vitro*.
27. The method of claim 23 wherein, the modulation of α -secretase reduces amyloid plaque formation and enhances cognitive ability in a patient with Alzheimer's Disease.
- 10 28. A method for treating Alzheimer's Disease comprising administering to a patient a macrocyclic lactone in an amount effective to treat Alzheimer's Disease and a pharmaceutically acceptable carrier.
29. A method for treating Alzheimer's Disease comprising administering bryostatin-1 to a patient in an amount effective to treat Alzheimer's Disease and a pharmaceutically acceptable carrier.
- 15 30. A method for enhancing cognitive ability in a human or animal, comprising administering to said human or animal a bryostatin or a neristatin class compound in an amount effective for enhancing cognitive ability in a pharmaceutically acceptable carrier.
- 20 31. A method for enhancing cognitive ability in a human or animal, comprising administering to said human or animal bryostatin-1 in an amount effective for enhancing cognitive ability in a pharmaceutically acceptable carrier.
32. A method comprising the modulation of α -secretase through the administration of a pharmaceutically effective amount of a bryostatin or a neristatin class compound and a pharmaceutically acceptable carrier.
- 25 33. A method for modulating α -secretase comprising administering bryostatin-1 in an amount effective to modulate α -secretase and a pharmaceutically acceptable carrier.

34. A method for providing a neuroprotective effect for cell comprising administering a bryostatin or neristatin class compound in an amount effective to provided a neuroprotective effect for cells which suffer from a hypoxic event and a pharmaceutically acceptable carrier.
- 5 35. A method for providing a neuroprotective effect for cell comprising administering bryostatin-1 in an amount effective to provided a neuroprotective effect for cells which suffer from a hypoxic event and a pharmaceutically acceptable carrier.
- 10 36. A method for the reduction of amyloid plaque formation comprising administering bryostatin-1 in an amount effective to reduce amyloid plaque formation and a pharmaceutically acceptable carrier.

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**FIG. 1A**

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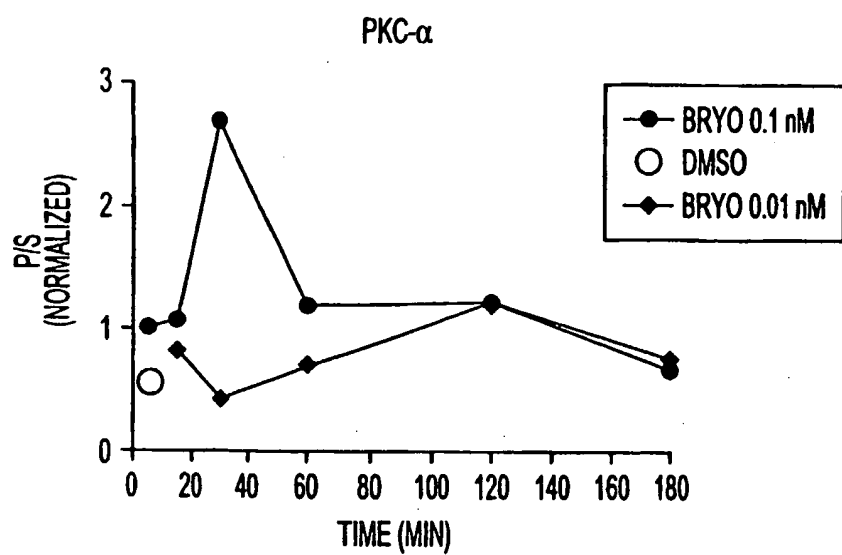


FIG. 1B

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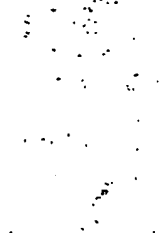
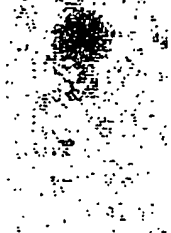
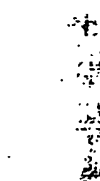
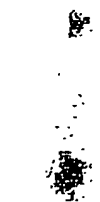
BL1.0µM

BL0.1mM

Sta-Bryo

Bryo

DMSO



AG06848 (AD)

AG07141 (AC)

FIG. 1C

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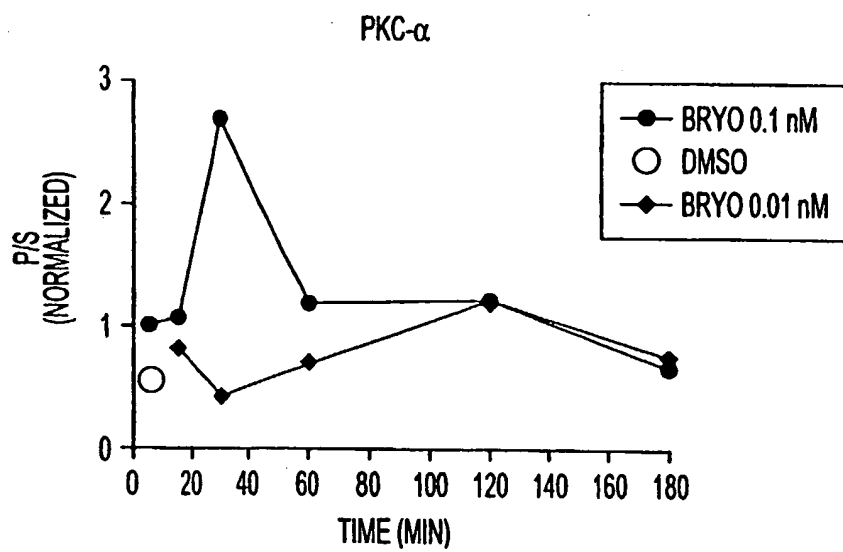


FIG. 2

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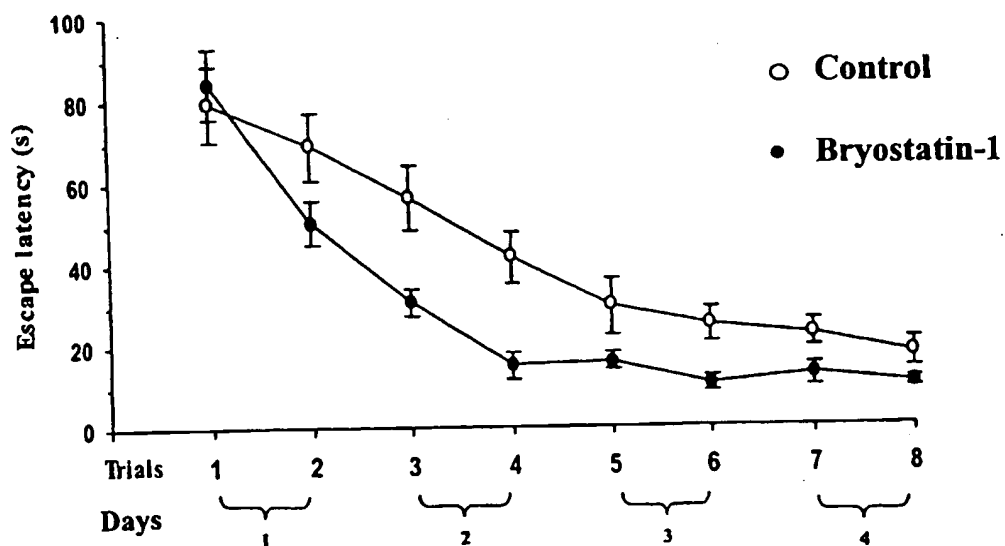


FIG. 3

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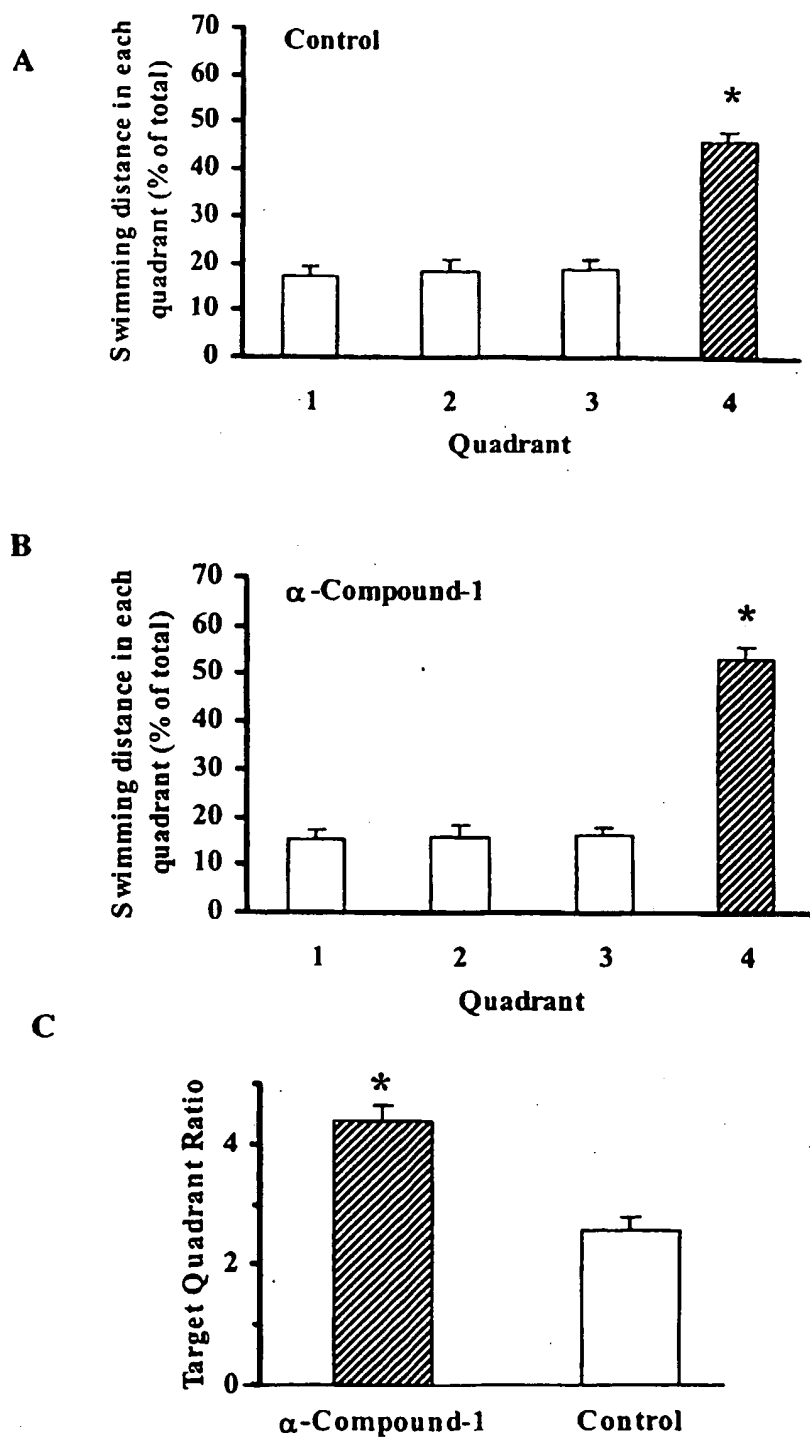
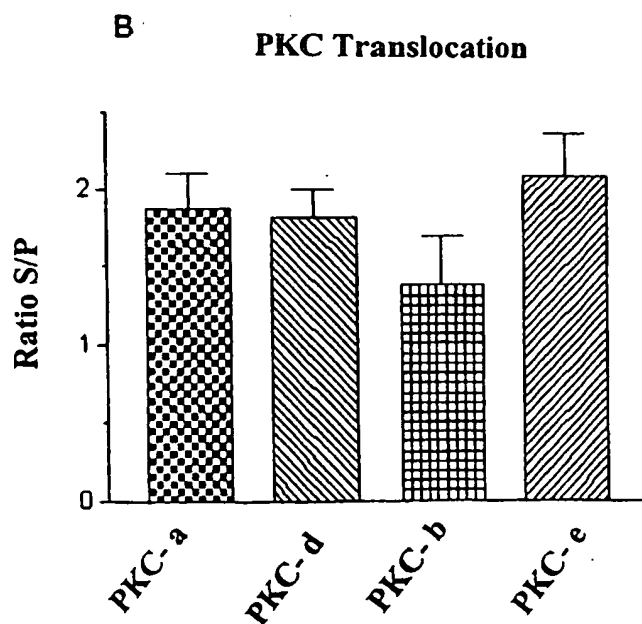
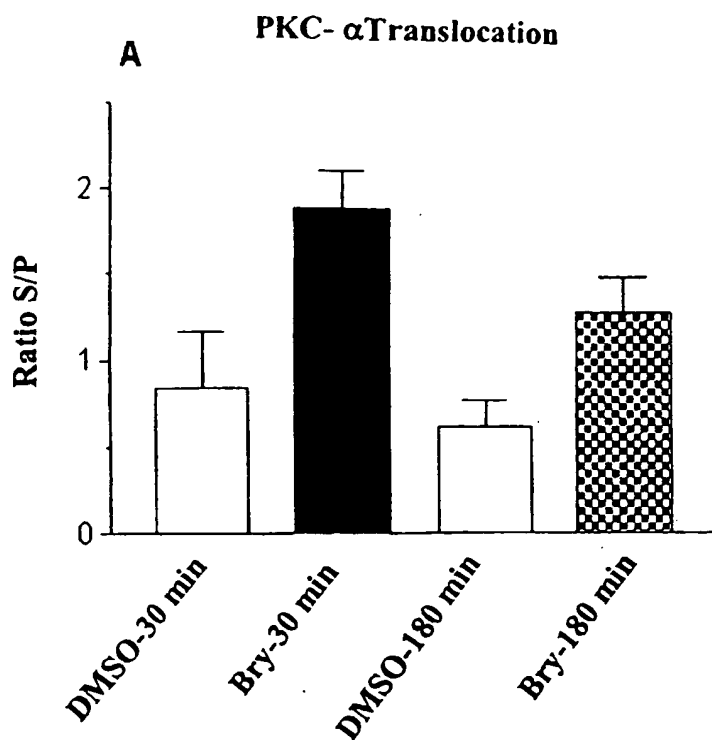
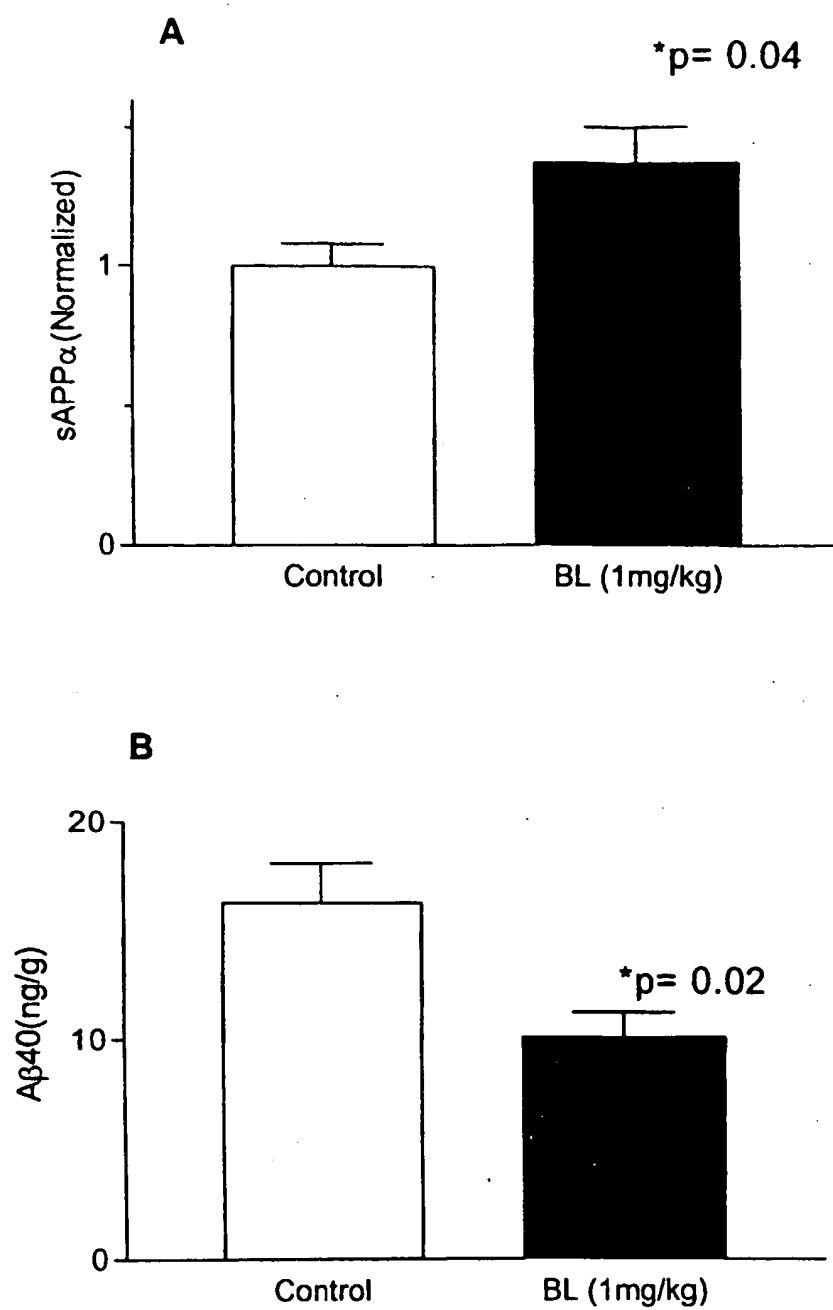


FIG. 4

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**FIG. 5**

SUBSTITUTE SHEET (RULE 26)

**FIG. 6**

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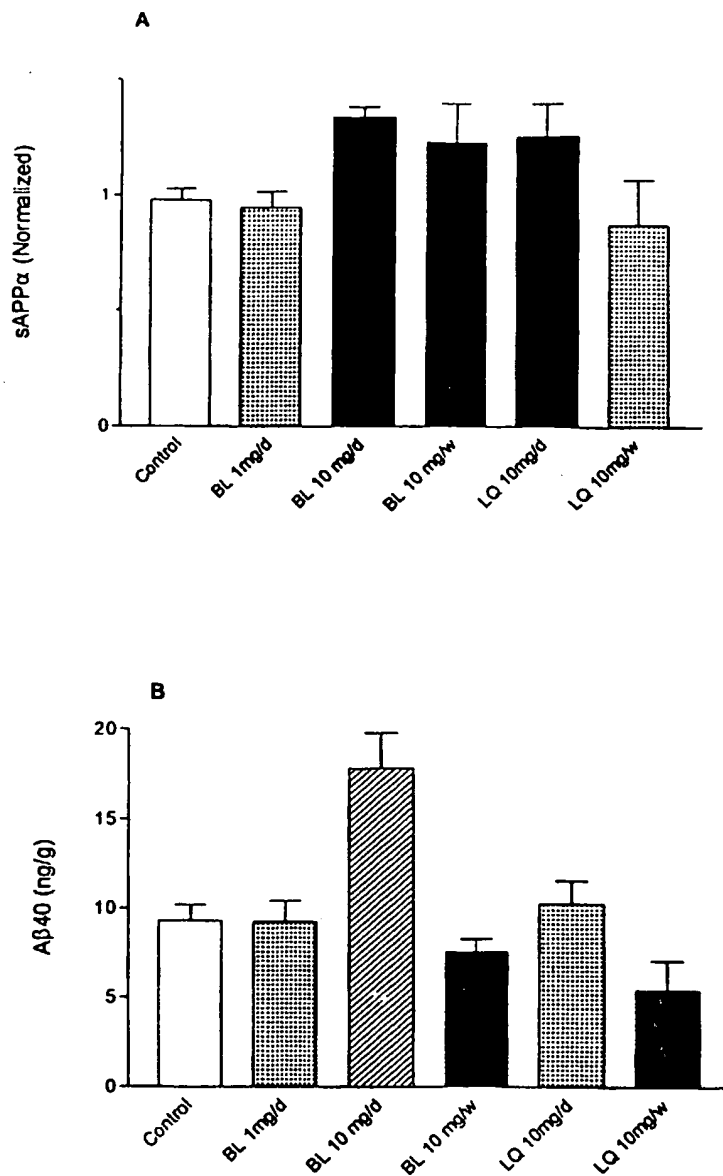
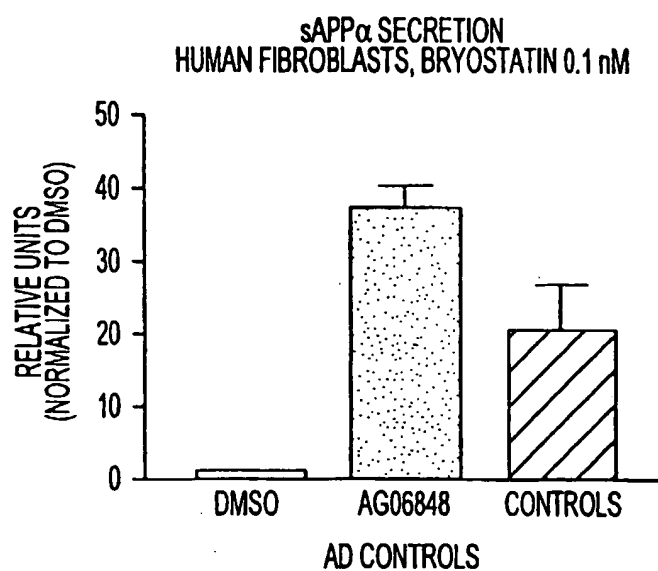
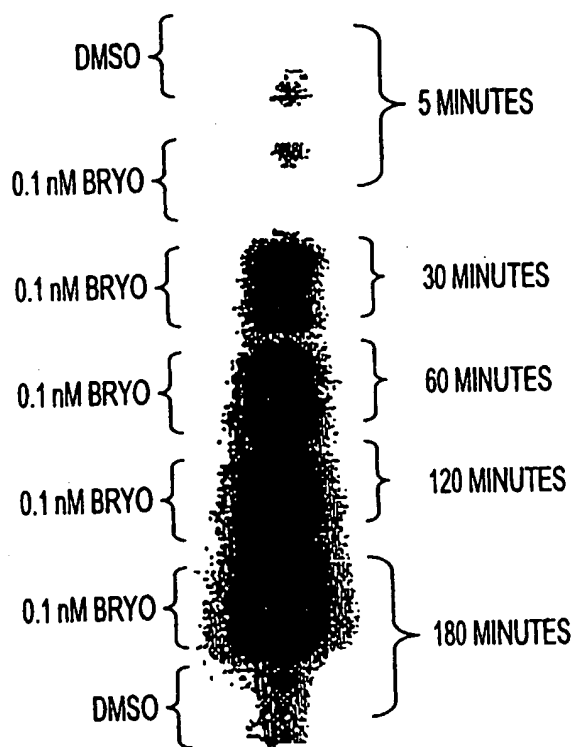


FIG. 7

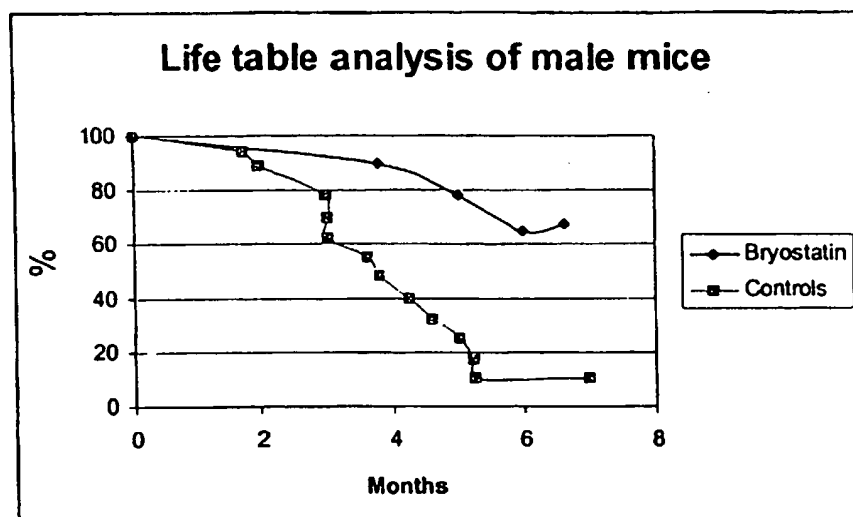
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**FIG. 8**

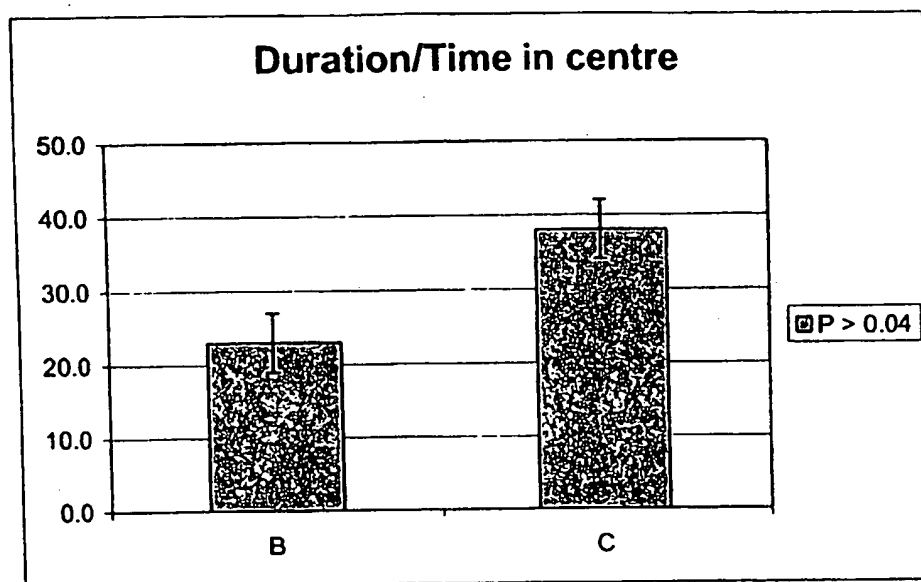
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**FIG. 9**

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**FIG. 10**

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**FIG. 11**

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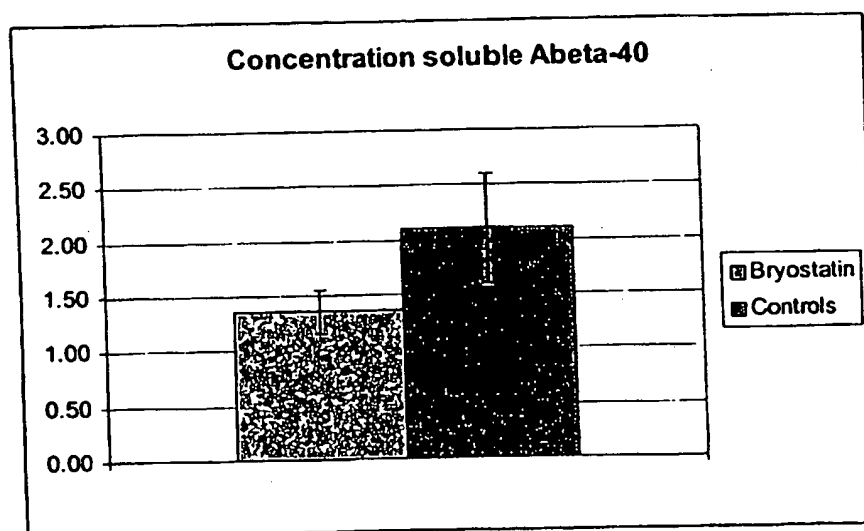
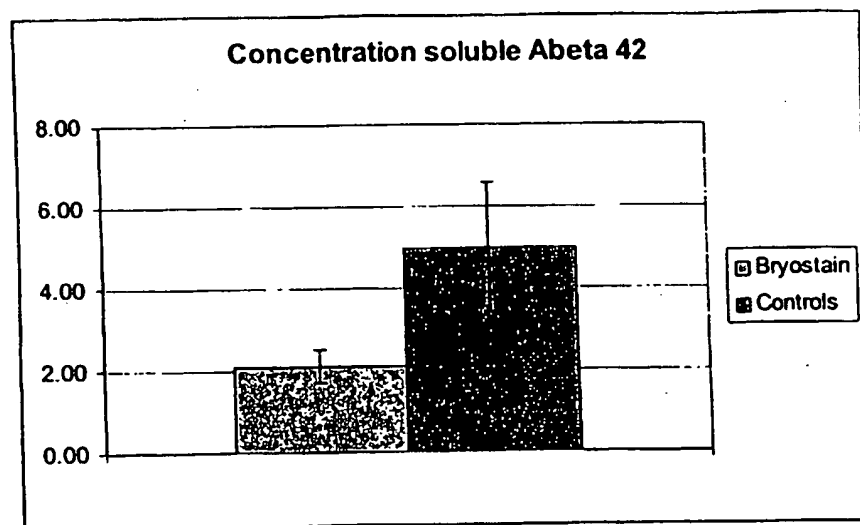


FIG. 12

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**FIG. 13**

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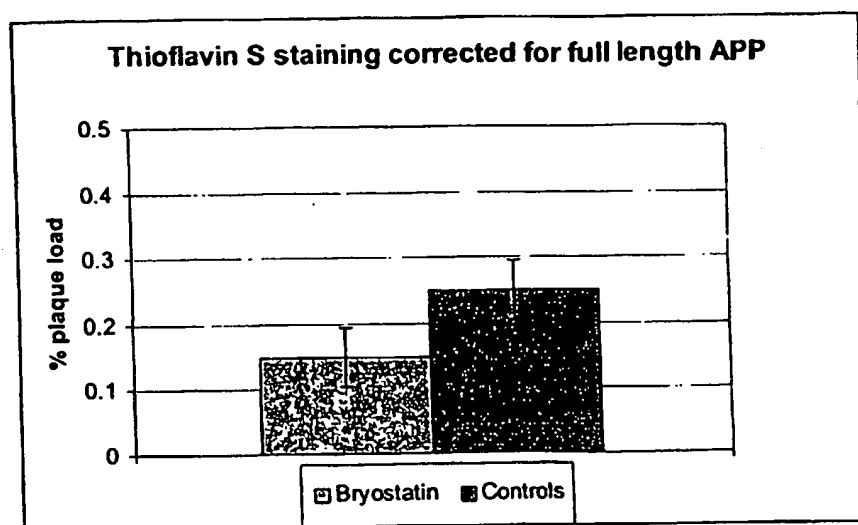


FIG. 14